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Fany Sardenne, Frédéric Ménard, Maxime Degroote, Edwin Fouché, Gaël Guillou, et al.. Methods of lipid-normalization for multi-tissue stable isotope analyses in tropical tuna. *Rapid Communications in Mass Spectrometry*, 2015, 29 (13), pp.1253-1267. 10.1002/rcm.7215 . hal-01168999

HAL Id: hal-01168999

<https://hal.science/hal-01168999>

Submitted on 26 Jun 2015

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Methods of lipid-normalization for multi-tissue stable isotope analyses in tropical tuna

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Key-words: Carbon, Nitrogen, C:N, lipid correction, large pelagic fish, trophic ecology.

Abstract

Rationale: The bias associated with lipid contents in fish tissues is a recalcitrant topic for trophic studies using stable isotopes. Lipids are depleted in heavy carbon isotope and lipid content varies considerably among species, tissues and in both time and space. We applied and assessed here different correction methods for tropical tuna tissues.

Methods: We tested two types of normalization methods to deal with variable lipid content in liver, gonads, white and red muscles of yellowfin, bigeye and skipjack tuna: a chemical extraction using dichloromethane and a mathematical correction based on three modeling approaches (linear, non-linear and mass balance models). We measured isotopic values of bulk and lipid-free tissues and assessed the predictive ability of the correction models with the lipid-free measurements. Models' parameters were estimated from our dataset and stemmed from published studies on other species.

Results: Comparison between bulk, lipid-free and lipid-corrected isotopic values demonstrated that (1) chemical extraction using dichloromethane did not affect $\delta^{15}\text{N}$ values; (2) change in $\delta^{13}\text{C}$ values after extraction was tissue-specific; (3) lipid-normalization models using published parameters' estimates failed to predict lipid-corrected $\delta^{13}\text{C}$ values; (4) linear and non linear models using parameters estimated for each tissue from our dataset provided accurate $\delta^{13}\text{C}$ predictions for all tissue, and mass balance model for white muscle only.

Conclusion: Models using published parameters' estimates from extra species cannot be used.

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35 According to a range of lipid content that do not exceed 45%, we recommend the linear model to correct bulk $\delta^{13}\text{C}$ values in the investigated tissues but parameters have to be estimated from a proportion of the original data for which chemical extraction is required and isotopic values of bulk and lipid-free tissues are measured.

For Peer Review

INTRODUCTION

Stable isotope analysis (SIA) is a widespread tool in trophic ecology, frequently used to understand how marine food webs are structured and how marine ecosystems function^[1]. The isotopic approach is based on a predictable relationship between a consumer and its preys, i.e. a stepwise enrichment in heavier isotope. With a low enrichment factor between trophic levels, carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$, expressed as $\delta^{13}\text{C}$) allow for discriminating different sources of primary production (e.g. nearshore *vs.* offshore systems, or benthic *vs.* pelagic systems) while nitrogen ratios ($^{15}\text{N}/^{14}\text{N}$, expressed as $\delta^{15}\text{N}$) provide an index of animal's trophic position due to preferential retention of ^{15}N during metabolism and excretion, and consequently a higher enrichment in $\delta^{15}\text{N}$ value between diet and consumer. Although the use of SIA has greatly improved the understanding of predator-prey interactions and trophic dynamics in marine environment, some limitations have also been pointed out because of ecosystem complexity, and variation in isotopic fractionation as well as tissue growth and isotopic turnover (e.g.,^[2–7]).

For the sake of standardization and of result comparability, trophic studies on fish using stable isotopes are recommended to be conducted on one particular tissue, usually white muscle due to its relative biochemical homogeneity and stability^[8]. In contrast, species-specific studies such as diet shift experiments have highlighted the strong need and interest to work on multiple tissues with different turnover rates^[9–13]. Beyond different turnover rates, tissue-specific isotopic signatures are also attributed to their biochemical composition, i.e., lipid and protein contents and their constituents fatty acids and amino acids^[14,15]. The tissue-specific allocation of these dietary components with different isotopic signatures largely influences the tissues' isotopic signatures (i.e., “isotopic routing”^[16,17]).

Lipids are depleted in ^{13}C relative to protein and glycogen due to the different biochemical pathways involved in their respective synthesis^[18]. Indeed the $\delta^{13}\text{C}$ value measured in a sample does not only reflect the diet of the individual, but is also related to the lipid composition of the tissue sample, which is known to vary considerably among tissues, individuals and species. Two different methods have been proposed to account for lipid influence on $\delta^{13}\text{C}$ value: either chemical extraction prior to SIA or mathematical correction of the bulk tissue values. The former successfully removes lipids from tissue samples and gives lipid-free $\delta^{13}\text{C}$ values. However, some chemical extraction method can affect $\delta^{15}\text{N}$ by removing some protein compounds linked to lipids (e.g., lipoproteins or transmembrane proteins). Two separate analyses for carbon and nitrogen stable isotopes is then required^[8,19–21], while some methods allow for simultaneous carbon and nitrogen SIA by using specific solvent mixtures; but they remain expensive and time consuming^[22–24]. The latter consists in mathematical models that predict lipid-corrected isotope values using SIA of bulk tissue and different covariates including a proxy of lipid content. Two main types of mathematical corrections

are currently available in the literature to predict the lipid-corrected isotopic values of carbon (Table 1): (i) statistical models using empirical relationships (linear or not) between bulk and lipid-free signatures; and (ii) the "mass balance" approach assuming that tissue are composed of lipid and protein only. There is no consensus for applying one particular type of correction model as evidenced by the large number of published studies addressing the question. Usually the most consistent models require species- or species-tissue-specific parameters.

In this paper, we dealt with the bias associated with variable lipid content in several tissues of three tuna species: bigeye tuna (*Thunnus obesus*; BET), skipjack tuna (*Katsuwonus pelamis*; SKJ) and yellowfin tuna (*T. albacares*; YFT). These large predatory fishes are widely exploited by industrial fisheries and their ecological role in trophic functioning of open ocean ecosystems still needs to be improved. During the last decade, several authors have applied SIA on the white muscle of tuna to investigate their trophic ecology^[34,46–49]. However, due to the high mobility of these large pelagic species and their opportunistic behavior (i.e., non-selective predation constrained by local prey availability and predator-prey size ratios^[50]), interpretation of isotopic data is not obvious. In parallel, multi-tissue analyses were developed in trophic studies of bluefin and yellowfin tuna to refine results about diet shifts^[13,46] but the issue of the influence of lipids on the tissue-specific isotopic signature has not been investigated in tropical tuna yet.

The central objective of this study was to deal with the highly variable lipid content in tuna tissues that impacts carbon isotopic values and interpretation, and then to assess the reliability of lipid removal using chemical extraction versus mathematical corrections that predict lipid-corrected $\delta^{13}\text{C}$ values. We therefore measured isotopic values of bulk and lipid-free white muscle, red muscle, liver and gonad tissues of SKJ, YFT and BET tuna caught in the Western Indian Ocean. We applied three types of mathematical corrections to the bulk measurements, with parameters either estimated from our data or selected in related studies, and assessed the predictive ability of the correction models with the lipid-free measurements. Then we discussed the relevance of mathematical normalization versus lipid removal. Finally, bulk and lipid-free isotopic signatures were discussed in terms of tissue-specific biochemical composition.

MATERIAL AND METHOD

Fish and tissue collection

A multi-species sampling was carried out from June 2012 to January 2014 during the unloading of the purse seiners at Victoria port (Seychelles) and was based on a 2-step methodology: (i) the ship wells were selected from among those containing fish from one set only, to obtain the exact fishing date and position, and (ii) fish from the three species of interest were chosen to sample the size range of purse-seine catches and to collect different life stages (i.e., immature and mature fish). A

total of 39 BET, 42 SKJ and 48 YFT were collected throughout the Western Indian Ocean, and processed at the laboratory. First, the total fish weight (W_T , kg), the fork length (F_L , cm), which refers to the length from the tip of the snout to the fork of the tail, and the sex were recorded for each individual. The weights of gonads and viscera (including stomach, liver, intestine, pyloric caecum, kidney, heart and spleen) were also determined and allowed for the estimation of the fish somatic weight (W_S : total fish weight minus viscera and gonad weights) and the gonadosomatic index (GSI: ratio between the gonad and somatic weights). Then, two samples of around 2g on a wet weight (ww) basis were taken from the dorsal white muscle (W; under the dorsal spine on the left side), red muscle (R; under the pectoral tail on the left side), liver (L) of each fish, as well as from gonads (G) for mature ones. All samples were stored at -80°C until further analyses.

Carbon and nitrogen stable isotope analysis

A total of 468 samples (129 L, 128 R, 129 W and 82 G) were freeze-dried to preserve the integrity of the tissues^[51]. Each dried sample was then ground up to a fine homogeneous powder with a mixer mill MM200 (Retsch, Eragny sur Oise, France), and split into two sub-samples: the first one was kept bulk in a dry room until encapsulation, while the second one was treated for lipid extraction.

Lipid extraction method

Lipids were extracted according to the method of Bodin *et al.*^[24]. Briefly, approximately $350 \pm 100\text{mg}$ of each powdered and dried sample was weighed and extracted with 8mL of dichloromethane (CH_2Cl_2) at 100°C under 1900psi for 10 minutes using a Dionex ASE 200 Accelerated Solvent Extractor (Voisins De Bretonneux, France). The lipid-free samples were dried under an extractor fan and stored in a dry room until encapsulation. The extracts were evaporated to dryness using a N-Evap 111 extractor (OA-SYS, Berlin, USA) at a bath temperature of 35°C and residues were weighed on an Adventurer Pro (OHAUS, Nänikon, Swiss) analytical balance to the nearest 0.1mg to determine the total lipid content of the samples expressed in % of dry weight (dw).

Isotope Ratio Mass Spectrometry

We weighed $0.4 \pm 0.1\text{mg}$ of each dried sample on a XP6 Metler Toledo microbalance (Viroflay, France) to the nearest μg and packed into $8 \times 5\text{mm}^2$ pressed tin capsules (Sylab, Metz, France) for simultaneous C and N SIA. Samples were analyzed using an Elemental Analyser (Flash EA 1112, Thermo Scientific, Milan, Italy) coupled to an Isotope Ratio Mass Spectrometer (Delta V Advantage with a Conflo iV interface, Thermo Scientific, Bremen, Germany) (EA-IRMS) at the LIENSs stable isotope facility (La Rochelle, France). Results were reported in the δ unit notation

and expressed as per mil (‰) relatively to international standards (Vienna-Pee Dee Belemnite for carbon and atmospheric N₂ for nitrogen). Calibration was done using reference materials (USGS-24, IAEA-CHE, -600 for carbon; IAEA-N2, -NO-3, -600 for nitrogen). Analytical precision based on replicate measurements of internal laboratory standard (acetanilide, Thermo Scientific) was < 0.15‰ for both δ¹⁵N and δ¹³C. C:N ratios were determined from % element weight. The extent of chemical extraction for lipid-free sub-samples was checked through the C:N ratio: 38 L and two G samples showed C:N ratios > 4 and were considered still fatty^[25]. These 40 samples were submitted to a second ASE extraction and EA-IRMS analysis as described above to prevent for any possible leftover lipids. The final C:N ratios, δ¹³C and δ¹⁵N values obtained were considered as lipid-free results. The total lipid content of these 40 samples corresponds to the sum of the masses of the two extracted materials.

Lipid class and protein analyses

A total of 219 samples (61 L, 63 R, 62 W and 33 G) were submitted to additional analyses to determine the tissue biochemical composition of the three tuna species. Approximately 250±50mg of each wet sample were weighed on an analytical balance to the nearest mg, then crushed using a FastPrep System® (MP Biomedicals, Illkirch, France) and extracted with 2mL of dichloromethane:methanol (2:1, v/v) (Sigma-Aldrich, St. Quentin Fallavier, France). After centrifugation the lower layer was collected then evaporated and re-suspended in a known volume of dichloromethane. An aliquot of the extract was spotted on chromarods SIII (rods of quartz covered with silica), and separated into lipid classes and quantified using an Iatroscan MK-6s (Mitsubishi Chemical Medience, Tokyo, Japan) thin-layer chromatography-flame ionization detector analyzer (TLC-FID)^[52]. Concentrations of triacylglycerols (TAG) and phospholipids (PL), the two main lipid classes in tropical tuna representative of reserve and structure lipids respectively^[53], were expressed in % wet weight (ww) using suitable calibration curves.

The total protein content was determined using bicinchoninic acid protein assay kit (BCA, Sigma-Aldrich, St. Quentin Fallavier, France). Approximately 15±5mg of each wet sample were weighed to the nearest mg, incubated with BCA after grinding in mixer mill MM400 (Retsch, Eragny sur Oise, France) and submitted to successive heat shocks (i.e., two passages of 30 seconds in liquid nitrogen and 5 minutes in ultrasonic baths at 30°C performed alternately). Absorbance at 520nm was determined using Infinite 200 spectrophotometer (TECAN, Lyon, France) and converted in protein concentration using Bovine Serum Albumine (BSA) as reference. Total protein contents were expressed in % ww.

Statistical analyses and models

Non parametric statistics (Wilcoxon's rank test and Spearman's rank correlation) were applied to our dataset.

General effects of lipid on SIA. The effect of lipid extraction on $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values was tested using paired Wilcoxon's tests (with V the Wilcoxon's statistic). The potential effect of explanatory variables (tissue, species, sex or lipid content) on differences between bulk and lipid-free $\delta^{13}\text{C}$ and then $\delta^{15}\text{N}$ values was assessed using linear regressions and Box-Cox transformations for variance stabilization. Indeed our sample size was large enough to consider that non-normality of residuals should not adversely affect the inferential procedures. Mean values were expressed ± 1 SD. The coefficient of variation (CV in %) was used for comparing range of variation. Hereafter the notations are: $\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{lipid-free}} - \delta^{15}\text{N}_{\text{bulk}}$ and $\Delta\delta^{13}\text{C} = \delta^{13}\text{C}_{\text{lipid-free}} - \delta^{13}\text{C}_{\text{bulk}}$.

Lipid-normalization modeling. Equations of lipid normalization models used C:N_{bulk}, lipid content and $\delta^{13}\text{C}_{\text{bulk}}$ to predict a lipid-corrected $\delta^{13}\text{C}$ value ($\delta^{13}\text{C}_{\text{corr}}$). Our data confirmed that bulk C:N ratio (C:N_{bulk}) was a reliable proxy of lipid content (for all tissues, Figure 1). Here we tested three models that include all types of lipid normalization models (Table 1): the non-linear model of McConnaughey and McRoy^[25] [eq1], the linear model of Post *et al.*^[32] [eq2], and the mass balance equation of Fry^[27] [eq3]:

$$[\text{eq1}] \quad \delta^{13}\text{C}_{\text{corr}} = \delta^{13}\text{C}_{\text{bulk}} + D \times \left(-0.207 + \frac{3.9}{1 + 287/L} \right)$$

$$[\text{eq2}] \quad \delta^{13}\text{C}_{\text{corr}} = \delta^{13}\text{C}_{\text{bulk}} + a \times C : N_{\text{bulk}} + b$$

$$[\text{eq3}] \quad \delta^{13}\text{C}_{\text{corr}} = \delta^{13}\text{C}_{\text{bulk}} + D - \left(\frac{D \times C : N_{\text{pureproteins}}}{C : N_{\text{bulk}}} \right)$$

Parameters are defined in Table 1. Models were first implemented using parameters based on our dataset. In [eq1] L was the lipid content from the ASE extraction of each sample, and in [eq3] the mean C:N_{lipid-free} for each tuna tissue was used as the C:N of pure proteins. Parameters a and b in [eq2], the lipid-protein discrimination D in [eq1] and [eq2] were estimated from our dataset and for each tissue using least-squares estimation.

Then models were implemented using parameters based on published studies. In [eq1] and [eq3] we used a value of 7.49‰ for D in muscles and 8‰ for liver and gonads based on Logan *et al.* investigations on bluefin tuna^[35]. I was fixed to -0.207 in [eq1] as in McConnaughey and McRoy^[25] and we adopted for a and b in [eq2] the value proposed by Post *et al.*^[32] for aquatic animals (0.99 and -3.32, respectively). To assess predictive performances, we trained each model with a random subset of the data (i.e., 2/3 of the data in each tissue). A cross validation was performed using the parameters estimated from the training data, in order to predict the unused data (validation dataset).

Besides we predicted six sets of lipid-corrected $\delta^{13}\text{C}$ data using models [eq1], [eq2] and [eq3] in both parameters' configuration. To assess the quality of each model, predicted values were compared to the observed data from the validation dataset, and differences were tested using paired Wilcoxon's test. Differences between the lipid-corrected and observed lipid-free $\delta^{13}\text{C}$ values are noted as $\Delta\delta^{13}\text{C}_{\text{error}}$ with $\Delta\delta^{13}\text{C}_{\text{error}} = \delta^{13}\text{C}_{\text{corr}} - \delta^{13}\text{C}_{\text{lipid-free}}$. The process of cross-validation on random subsets was repeated 500 times. Thereby paired Wilcoxon's tests were carried out for each model and at each trial, and means, maxima and distributions of $\Delta\delta^{13}\text{C}_{\text{error}}$ were computed or plotted to detect any mismatch.

Lipid class composition and total protein content. The effects of tissue and sex on lipid class concentrations and total protein content were tested for each tuna species using Wilcoxon's test. Ranges of variation were compared using CV. Statistical analyses were performed using R 3.0.2 software^[54]. Function *nls* of library MASS was used for nonlinear least-squares estimates.

RESULTS

Morphological characteristics (F_L and W_T) of the sampled tuna as well as their tissue isotopic composition and total lipid content are presented in Table 2.

Bulk tissue characteristics

Lipid and protein composition. The total lipid and the protein contents varied among tissues and species. Liver was on average the fattest and white muscle the leanest somatic tissue for all species and lipid content was similar in red muscle and in gonads ($V=5600$, $p=0.41$). SKJ showed the lowest lipid variation in liver (CV = 36.3%, min-max = 8.1-30.4% dw) and in white muscle (25.4%, 1.08-3.6% dw). For YFT, CV was 57% in liver (2.7-43.3% dw) and 45.8% in white muscle (0.3-5.7% dw). For BET, CV was 49% in liver (6.6-40% dw) and 65% in white muscle (1.2-8.8% dw). Red muscle showed the largest lipid variation in BET (45.2%, 3.1-26.2% dw) compared to YFT (30.4%, 5.5-19.3% dw) and SKJ (29.6%, 4.4-18.1% dw). Gonads of SKJ had a smaller range of variation in lipid content (21.2%) compared to BET (63.4%) and YFT (60.6%). In addition, GSI of mature tuna was correlated with the lipid content of gonads (Spearman's statistic $S=56408$, $p<0.001$) only (liver: $S=117758$, $p=0.15$; red muscle: $S=150494$, $p=0.24$; white muscle: $S=144226$, $p=0.69$). For all species, liver had higher protein levels than white muscle ($V=2813$, $p<0.01$) and red muscle ($V=5289$, $p<0.001$). Finally, very few reserve (TAG) lipids were recovered in white muscle of the three species (Figure 2), while red muscle and liver, which had higher contents than white muscle (liver: $V=3646.5$, $p<0.001$ and red muscle: $V=4020$, $p<0.001$), did not

differ significantly ($V=2403$, $p=0.76$). Structural lipids (PL) were higher in red muscle than in liver ($V=3259$, $p<0.01$), and higher in liver than in white muscle ($V=3668$, $p<0.001$). TAG and proteins contents in gonad of the three species were higher in female than in male ($V=236$, $p<0.01$ and $V=319$, $p<0.01$, respectively) but differences were not significant for PL ($V=135$, $p=0.77$).

$\delta^{15}\text{N}$, $\delta^{13}\text{C}$ values and C:N ratios. Bulk $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values varied between tissue and species. Mean bulk $\delta^{15}\text{N}$ values ranked BET > YFT > SKJ in all tissues. Liver (9%), white muscle (8.9%), gonads (8.5%) and red muscle (7.8%) had very close CV of bulk $\delta^{15}\text{N}$ values, and, overall, YFT exhibited the greatest CV and BET the lowest. White and red muscles had low CV of bulk $\delta^{13}\text{C}$ values (2.4 and 2.7%, respectively), while liver and gonads showed higher but similar CV (4.5 and 4.7%, respectively). Finally, bulk C:N ratios varied according to lipid content variation: 21.6% in liver, 13.4% in gonads, 7.4% in red muscle and only 2.2% in the leanest tissue, the white muscle. Interestingly, bulk C:N ratios significantly differed between male and female in SKJ gonads only ($V=236$, $p<0.001$), with higher values in females (mean C:N > 4).

Influence of lipid extraction on tissue isotopic composition

Effect of ASE extraction method. Most of the samples showed a C:N ratio < 4 after a first lipid extraction, except for 40 tissues characterized by higher total lipid contents (18.5 ± 7.7 % dw). A second extraction was then processed, which removed on average 2% dw more of lipids (between 0.5 and 10%). The repeated extraction did not significantly affect $\delta^{15}\text{N}$ values ($V=481$, $p=0.21$), but it slightly increased $\delta^{13}\text{C}$ values (0.1‰ in mean, $V=585$, $p<0.05$) and surprisingly the C:N ratio (0.08 in mean, $V=67$, $p<0.001$) (Table 3). Despite the two chemical extractions performed successively, 19 samples were still characterized by C:N ratio > 4.

$\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ changes after lipid extraction. A tiny but significant increase of $\delta^{15}\text{N}$ was observed after lipid extraction ($\Delta\delta^{15}\text{N} = 0.1\pm 0.2\text{‰}$; $V=85992$, $p<0.001$). A tissue effect was detected at the 5% significance level by the regression linear applied to $\Delta\delta^{15}\text{N}$ values (Table 4). Obviously $\delta^{13}\text{C}$ values increased strongly after lipid extraction ($V=108567$, $p<0.001$). Linear regression results showed that lipid content, tissue and sex affected $\Delta\delta^{13}\text{C}$ values (Table 4). The highest change in $\delta^{13}\text{C}$ value was observed in liver ($\Delta\delta^{13}\text{C} = 1.6\pm 0.4\text{‰}$) and the lowest in white muscle ($\Delta\delta^{13}\text{C} = 0.1\pm 0.1\text{‰}$). The variability of the $\delta^{13}\text{C}$ values was clearly reduced in all tissues after the lipid extraction: CV decreased from 4.8 to 3.6% in gonads, from 4.5 to 2.5% in liver and from 2.7 and 2.4% to 2.3 and 2.1% in red and white muscles, respectively. Gonads were the only tissue with differences between male and female: C:N_{lipid-free} ratios measured in gonads were positively correlated to GSI in females for the three species (BET: $V=144$, $p<0.001$; SKJ: $V=186$, $p<0.01$ and

YFT: $V=121$, $p<0.001$) and negatively in males (BET: $V=81$, $p<0.001$; SKJ: $V=188$, $p<0.05$ and YFT: $V=361$, $p<0.001$) (Figure 3). Finally C:N_{lipid-free} ratios were less variable in muscles (CV=1.3 and 3.7% in white and red muscles, respectively) than in liver and gonads (9.6 and 9.2%, respectively).

Lipid correction models for $\delta^{13}\text{C}$ values

As tissue impacted lipid content and bulk C:N ratios, models were fitted for each tissue. Indeed our results did not justify species- or sex-specific models.

We then assessed the predictive performance of the three models in the two parameters' configuration: based on our dataset and based on published studies. Table 5 presents the estimated values of parameters for each tissue and from our dataset, and Table 6 displays the results of the cross validation procedure that was repeated 500 times. Results of the paired Wilcoxon's tests between predicted and observed values were expressed in % of acceptable p -values (i.e., % of $p>0.05$) among the 500 iterations. The three models [eq1], [eq2] and [eq3] using parameters based on published studies gave very bad predictions with systematic discrepancies with $\delta^{13}\text{C}_{\text{lipid-free}}$. Figure 4 displays the distribution of $\Delta\delta^{13}\text{C}_{\text{error}}$ values computed from the three models using parameters from our dataset. For white muscle, the three models provided reliable predictions and were suitable for correcting bulk $\delta^{13}\text{C}$ values. Again [eq1] and [eq2] gave accurate predictions for the three other tissues even if maximum differences between $\delta^{13}\text{C}_{\text{corr}}$ and $\delta^{13}\text{C}_{\text{lipid-free}}$ could occasionally be much greater than 1‰. However [eq3] did not provide the same level of good predictions compared to the other models: systematic mismatch was observed in the distribution of the $\Delta\delta^{13}\text{C}_{\text{error}}$ values showing an underestimation of predicted values (Figure 4).

DISCUSSION

Lipids greatly influenced the carbon isotopic composition of soft tissues of tropical tuna as evidenced by the significant increase in $\delta^{13}\text{C}$ after lipid extraction, especially in fat tissues such as liver, gonads and red muscle (Table 2). As lipid composition varies among tissues, species and individuals (related to life cycle, i.e. reproduction, sex, age, migration, starvation and/or to environmental factors), lipid normalization techniques are required for interpreting carbon isotopic composition. The lipid extraction method using dichloromethane only, as proposed by Bodin *et al.*^[24], had a negligible effect on $\delta^{15}\text{N}$ values (increase of 0.1‰ in mean) across tissues and tuna species (Table 2). This modification is under the IRMS precision (0.15‰) confirming that distinct analyses of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are not required for tropical tuna tissues with this chemical extraction. Moreover, the low total lipid content found in white muscle (less than 3% dw, Table 2) and its composition (proteins and polar lipids, Figure 2) revealed that lipid removal prior to SIA is

not essential for this tissue. For the fattest tissues (i.e., liver and possibly gonads), however, a longer ASE extraction process using maybe more solvent compared to the method of Bodin *et al.*^[24] might be necessary to remove properly most of lipid compounds.

The liver of topical tuna was characterized by an high C:N ratio ($C:N = 5.7 \pm 0.3$) even after two ASE extraction cycles. A similar result was previously observed in sea bass liver ($C:N$ ratio = $5.6^{[30]}$) and could reflect the peculiar C:N signature of proteins in tuna liver. But this high ratio could also relate to highly polar lipids that were not totally extracted with the method of Bodin *et al.*^[24]. Indeed, by using a medium polar solvent (dichloromethane with dipole moment of 1.6 D), this method removes neutral and medium polar lipid compounds but not the most polar ones (e.g. some phospholipids or lipoproteins) in order to avoid any unwanted effect on $\delta^{15}N$ value^[24]. The additional biochemical analyses performed in the present study revealed the high quantity of proteins and phospholipids in the liver of tropical tuna (Figure 2), and therefore strengthened the second hypothesis that the observed high C:N ratio in the ASE-extracted liver reflected the signature of proteins and remaining highly polar lipids.

Lipid correction models

To overcome a tedious, time-consuming, and costly chemical extraction process, we investigated different mathematical models that deal with lipid contents and predict lipid-corrected isotopic values using SIA of the bulk tissue and different covariates. We analyzed several tissues of three tropical tuna species and estimated specific parameters with our dataset. The confidence of the tissue-specific models was thoroughly assessed using a cross validation procedure that tested their predictive ability. Models using published parameters' estimates from other species systematically failed to predict lipid-corrected $\delta^{13}C$ values. Such approaches are definitively not suited for trophic studies using SIA. Linear and non-linear models (i.e., equations of Post *et al.*^[32] and McConnaughey and McRoy^[25], respectively) provided accurate $\delta^{13}C$ predictions when parameters were estimated for each tissue and from our dataset. The mass balance equation of Fry^[27] gave reliable predictions for white muscle only, which is the leanest tissue (i.e., lipid content $<10\%$ dw). But the mass balance equation led to systematic biased predictions for red muscle, liver and gonads: predicted values were underestimated and a larger mismatch was observed for gonads (mean $\Delta\delta^{13}C_{\text{error}} = -0.28$) and liver (mean $\Delta\delta^{13}C_{\text{error}} = -0.18$) than for red muscle (mean $\Delta\delta^{13}C_{\text{error}} = -0.05$) (Figure 4). For red muscle, liver and gonads, discrepancies between lipid-free and corrected values of a given model could be great. The maximum $\Delta\delta^{13}C_{\text{error}}$ ranged from 1.2% in red muscle with [eq2] and [eq3] to more than 1.6% in liver with [eq1] and 1.6% in gonads with [eq3]. Despite these discrepancies, [eq1] and [eq2] provided accurate predictions in all tissue and in about 90% of the time. Therefore they can be used for correcting bulk $\delta^{13}C$ values in trophic studies according to a

close range of lipid content that we measured in our tuna tissues. But we do not recommend [eq3] that gave systematic biased predictions for red muscle, liver and gonads, despite errors within an acceptable range for ecological interpretations. We suspected that this result relied on the correct estimation of $C:N_{\text{lipid-free}}$ as previously observed^[30]. Indeed discrepancies between lipid-free and corrected values increased with the $C:N_{\text{lipid-free}}$ variability: 54% of good prediction for red muscles with a $C:N_{\text{lipid-free}}$ variability around 3.6%, which went down to 10-1720% in gonads and liver with a $C:N_{\text{lipid-free}}$ close to 9%.

However all models did not required the same level of information. The equations of McConnaughey and McRoy^[25] (i.e. [eq1]) and the mass balance approach of Fry^[27] (i.e. [eq3]) required empirical information in addition to the estimation of parameters from a part of the dataset: the lipid content L , which was sample dependant for [eq1], and the $C:N$ of pure proteins that varied among tissue and needed lipid-free samples for [eq3]. Moreover adjusted parameters were also tissue-specific. For instance D , which was estimated from our dataset, varied among tissue in [eq1] mainly and in [eq3] to a lesser extent, and between [eq1] and [eq3] (Table 5), suggesting that D is not constant but could vary according to tissue composition. The highest D values were estimated in liver for both [eq1] and [eq2], but no clear trend was observed between D estimates and the mean total lipid nor between D estimates and lipid classes (TAG and PL) of each tissue. Further biochemical analyzes involving for instance the dynamics of fatty acids (see below) are needed to understand the processes of lipid-protein discrimination. The estimation process might weaken here the basic assumptions of biochemical composition used in the mass balance approach. Finally [eq2] was easier to implement as this model did not require lipid content and thus we recommend its use for tropical tuna according to the range of lipid content we measured in the tissues under study. Therefore correction models used to mathematically normalize $\delta^{13}C$ values for lipid contents require a proportion of the original dataset for estimating parameters specific to tissue, while chemical extraction of lipids necessitates the process of all samples. Here we combined data of the three tuna species despite the fact that some differences were highlighted by statistical tests. We then increased the amount of data and improved the precision of the parameters' estimation process. Species- or sex-specific models (results not shown) did not make better the predictions and led to very close parameters' values confirming that tissue is the most important factor to consider in lipid correction models, as already noted by Logan *et al.*^[35]. However extrapolation to other species or extra tissues should not be performed without estimating specific parameters.

As already mentioned, white muscle of BET, SKJ and YFT did not require lipid removal and any correction model would provide a suitable correction for trophic investigations, regarding the small difference between bulk and lipid-free $\delta^{13}C$ values (around 0.1‰). A mean error of more or less

0.1‰ in $\delta^{13}\text{C}$ values would not alter ecological interpretation and is in the range of the measurement error of the device as well. However, when multi-tissue SIA are carried out using fatter tissues or when comparative studies aim at investigating the role and position of different species in marine food webs, samples have to be processed in the same way, i.e. chemical lipid extraction or normalization using specific mathematical models. Although expensive, chemical extraction has the advantage to gain additional valuable information such as the total lipid content of tissues, which is commonly used as a proxy of nutritional and/or energetic condition of fish^[55–57].

390 Tissue metabolism and isotopic composition

Strong differences were observed in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values among the tissues of the three tuna species. Tissues showed indeed a large variability in lipid and protein contents, and metabolism affects most likely isotopic composition. Hereafter the objective is not to interpret our findings in a trophic context but to discuss physiological factors that could influence our isotopic results. Among the two types of tuna muscles, red muscle accounts for less than around 10% of the total body weight, depending on the fish species and size^[58]. Indeed, due to increased endothermic efficiency with age, the proportion of red muscle tends to decrease^[58]. Red and white muscles have different functions in tuna. White muscle is activated during fast and short-duration swimming bursts fueled by catabolized glycogen and anaerobic metabolism. In contrast red muscle is activated during routine swimming and is based on aerobic lipids catabolism (i.e. fatty acid degradation for energy production)^[59,60]. Indeed, storage lipids are rather found in red muscle compared to white muscle (Figure 2). Catabolism preferentially uses and excretes light isotopes. In the case of red and white muscles, catabolized substrates have different isotopic signatures: lipids are depleted in $\delta^{13}\text{C}$ relatively to glycogen^[18]. This could explain the lower $\delta^{13}\text{C}$ values observed in the red muscle compared to white muscle in this study.

However, lipid metabolism could also have specific preferences that will influence the isotopic composition. Sidell *et al.*^[61] noted that some monounsaturated fatty acids are preferentially used for energy metabolism in the muscle of an Antarctic fish. As fatty acids could have different $\delta^{13}\text{C}$ values according to their origins^[62], the resulting carbon pool could be differentially affected. In addition, Koussoroplis *et al.*^[63] showed that fatty acids from structural (PL) or storage (TAG) lipids have different $\delta^{13}\text{C}$ values in the grey mullet, thus proportion and use of each lipid class could influence isotopic composition of each tissue. Similarly, McMahon *et al.*^[64] showed that fish muscle $\delta^{13}\text{C}$ values is greatly influenced by the amino acids available in the diet and by the metabolic ways activated to synthesize the missing ones.

Nitrogen isotopic composition depends on the amino acid metabolism through deamination and transamination processes^[65]. Tissues with high protein turnover (i.e., ratio between protein

degradation and synthesis) excrete lighter nitrogen leading to a ^{15}N -enrichment. In this way, higher $\delta^{15}\text{N}$ values are expected in liver which is characterized by faster turnover than muscles in fish (e.g.^[9,12, 66]). However, in tropical tuna, the lower $\delta^{15}\text{N}$ values observed in liver compared to muscle^[46, this study] suggest that other factors are at play such as tissue growth (higher proteins synthesis) or tissue specific metabolism. Indeed liver contains high levels of proteins and plays a central function in amino acid and fatty acid metabolism and their exchange with other tissues^[67]. Its isotopic composition should be greatly influenced by compounds brought by diet or their synthesis and degradation for the organism requirement. Lower $\delta^{15}\text{N}$ values could also suggest low reuse of proteins on site, thus protein are rapidly transferred in other tissues. Details in essential and non-essential amino acid and fatty acid proportions would improve our conclusions in terms of compound distribution from the liver to tissues.

On the other hand, in the red muscle of teleost fish, glutamine, a non-essential amino acid, is catabolised by mitochondria “on site” (not in liver like in mammals) in addition to fatty acid metabolism^[68,69]. This characteristic of red muscle could explain its high $\delta^{15}\text{N}$ values although it contains lower proportion of proteins compared to other tissues (Figure 2). The differences of total protein contents between the two types of muscle may be linked with their myofibrillar protein levels: while muscle cells typically contain a high quantity of these compounds, red muscle is characterized by a lower fiber diameter and thus a lower myofibrillar protein level to ensure the efficiency of its large number of mitochondria^[60,70]. Starvation may also cause a tissue ^{15}N enrichment but these situations seems unlikely in tuna who have high energetic requirements: experiments on SKJ and YFT have actually disclosed a limited ability to survive starvation^[71].

Gonads were the only tissue that showed sex difference in isotopic composition: C:N ratio increased with maturity (i.e, GSI) in females. However the decrease in male gonads is constrained by C:N ratio in non mature males (i.e., GSI < 1.5, Figure 3). The variation in proximal composition supports this pattern: proteins and reserve lipids were more abundant in female gonads of BET and SKJ (Figure 2) and this is probably linked to the different requirements between ovary and testis during maturation process. In females, liver products vitellogenin (Vtg), a large glycolipoprotein intended to energy storage in eggs. A greater Vtg accumulation in bluefin tuna *Thunnus thynnus* was suggested to be correlated with diet quality and especially with diet dominated by squid, which are rich in protein and phospholipids^[72]. Thus diet quality could influence the ovary isotopic composition. Further studies suggest also that oleic acid, a specific fatty acid, is preferentially used in females for the yolk supply in fish^[73,74]. High levels of PolyUnsaturated Fatty Acids (PUFA) in eggs are also correlated with better quality and fecundity in many fish species^[75,76]. By this way, maternal investment could also affect ovary isotopic composition. But sperm is rich in ATP and glucose, which are essential for its mobility. The nucleotide concentration (ATP-ADP-AMP) could

therefore be used as an indicator of sperm quality^[77]. Many differences of compounds during maturation could thereby explain differences in isotopic composition among sexes.

Moreover, differences in gonad $\delta^{13}\text{C}$ values were also observed among tuna species. Indeed different reproductive strategies are observed for the three species^[78-82]. The reproductive investment of SKJ females mobilizes more energy storage by means of lipid than males, which is common in fish. However more analyses on energy allocation strategy of these species are required to better understand the isotopic routing, and thus the isotopic composition of ovary and testis. Lastly our results showed that the reproductive investment of the three species did not truly depend on the energy storage in the other tissues, as variation in isotopic or in biochemical compositions with GSI were observed in gonads only. The understanding of isotopic routing is an important step required to improve multi-tissue isotopic studies. Indeed the multi-tissue approach promises interesting results on tropical tuna. For example, Graham *et al.*^[46] detected a diet shift at 45-50cm FL in Pacific YFT by comparing isotopic results in muscle and liver on the one hand and stomach contents on the other hand.

Conclusion

Mathematical corrections of lipid content can be applied on bulk $\delta^{13}\text{C}$ values of white and red muscles, liver and gonads of yellowfin, bigeye and skipjack tuna. However parameters have to be estimated from a proportion of the original data for which chemical extraction is required and isotopic values of bulk and lipid-free tissues are measured. On the one hand, we recommend using the linear model of Post *et al.*^[32] for tuna tissues with a similar range of lipid content (0.3-45%), as it was the most simple to implement with our dataset. On the other hand the mass balance approach was not suitable for correcting bulk $\delta^{13}\text{C}$ values of red muscle, liver and gonads of tuna. Models using published parameters' estimates from extra species cannot be used to account for lipid correction in trophic studies using SIA. Multi-tissue stable isotope analyses help to disentangle the complex relations between diet, physiological condition (growth, reproduction, starvation...) and the tissue-specific metabolism. The proportions of essential and non-essential fatty acids and amino acids metabolized in fish tissues shape isotopic composition, and compound-specific isotopic analyses would improve our understanding of tissue metabolism.

Acknowledgements

We are grateful all the SFA technicians lab and in particular Maria Cedras, Greg Burke and Rodney Melanie for their help throughout the tuna sampling. We wish also to thank the contributions of the IRD/SFA/IEO samplers, purse-seine fishermen, and of Patrice Dewals and Emmanuel Chassot of

the IRD Tuna Observatory (UMR MARBEC, Seychelles). We thank also Hervé Guillou and his team (UMR TOXALIM, Toulouse, France) for their help in the preparation of the samples of lipid class and protein analyses. Finally we thank three anonymous referees for their remarks and suggestions that greatly improved the manuscript. This work is a contribution to projects EMOTION, CANAL and ALECAP, respectively founded by ANR, MW Brands IOT Ltd. and France Filière Pêche.

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Figure caption

Figure 1. Relationship between bulk C:N ratio and total lipid content (% dw) for each tissue. A curvilinear model was significant in white muscle only. Coefficients of determination (R^2) are given.

505 **Figure 2.** Lipid and protein composition (in % ww) of the white muscle, red muscle, liver and gonads of the three tropical tuna species. Reserve lipids refer to TriAcylGlycerol (TAG) and structural lipids to Phospholipids (PL). Gonad F = female gonads; Gonad M = male gonads.

Figure 3. Linear regression models between lipid-free C:N ratio and the Gonado-Somatic Index (GSI) in gonads of males and females of the three tropical tuna species.

510 **Figure 4.** Distributions of the $\Delta\delta^{13}\text{C}_{\text{error}}$, i.e. the differences between the lipid-corrected and observed lipid-free $\delta^{13}\text{C}$ values. Lipid-corrected values were predicted from the three lipid-normalization models using a cross validation procedure that was repeated 500 times. $\Delta\delta^{13}\text{C}_{\text{error}} = \delta^{13}\text{C}_{\text{corrected}} - \delta^{13}\text{C}_{\text{lipid-free}}$

515 **Table caption**

Table 1. Non-exhaustive inventory of lipid-correction models from the literature and developed for marine organisms. Many studies compared different models using their data and are cited in the corresponding model type.

520 **Table 2.** Mean values (\pm standard deviation) of total lipid content (% dw), isotopic data ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) and C:N ratios for bulk and lipid-free samples in the gonads (G), liver (L), red muscle (R) and white muscle (W) of tropical tuna (YFT=yellowfin; BET=bigeye; SKJ=skipjack) sampled in the Western Indian Ocean.

525 **Table 3.** Results of the two chemical extractions performed successively on 40 samples: mean values (\pm standard deviation) of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, and C:N ratio of bulk samples and after chemical extractions. Letters indicate significant differences ($p < 0.05$) based on Wilcoxon's test: a = different from bulk, b = different from first extraction.

530 **Table 4.** Diagnostic statistics of the linear regression models with $\Delta\delta^{15}\text{N}$ and $\Delta\delta^{13}\text{C}$ as dependant variables, and lipid content, tissue, species and sex as explanatory variables. $\Delta\delta^{15}\text{N} = \delta^{15}\text{N}_{\text{lipid-free}} - \delta^{15}\text{N}_{\text{bulk}}$ and $\Delta\delta^{13}\text{C} = \delta^{13}\text{C}_{\text{lipid-free}} - \delta^{13}\text{C}_{\text{bulk}}$, i.e. the differences between the isotopic values of bulk and lipid-free samples.

Table 5. Estimated parameter values (\pm standard deviation) from our dataset of the three correction models that predict lipid-corrected $\delta^{13}\text{C}$ values (see text).

Table 6. Assessment of the prediction of the three models [eq1], [eq2], [eq3] using a cross validation procedure that was repeated 500 times, and in the two parameters' configuration: based on our dataset (Table 4) and based on published studies (see text). Paired Wilcoxon's tests were carried out for each model and at each trial to compare predicted ($\delta^{13}\text{C}_{\text{corr}}$) and observed ($\delta^{13}\text{C}_{\text{lipid-free}}$) values. Good predictions are expressed in % of acceptable p -values (i.e., % of p -values > 0.05) for the 500 iterations. Mean and maximum values of $\Delta\delta^{13}\text{C}_{\text{error}} = \delta^{13}\text{C}_{\text{corrected}} - \delta^{13}\text{C}_{\text{lipid-free}}$ are specified.

	Taxon	Tissue	N	Bulk C:N (min-max)	Ref.
$\left(I + \frac{3.9}{1 + 287/L} \right)$ measured or calculated as	Bering sea organisms (plankton to mammals)	Various	75	3.2-9.2	25
	14 fishes / 5 invertebrates	Muscle / Whole	227	2.9-9.4	29
	Zooplankton spp.	Whole	120	4.8-17.8	33
	2 seabirds	Muscle / Liver / Feathers	94	3.2-7.1	34
	2 antarctic fishes	Muscle	20	3.6-7.1	36
	16 fishes / 10 invertebrates	Muscle / Liver / Gonad / Whole	431	2.5-12.5	35
	2 cormorants	Muscle / Liver	137	4.4-5.5	37
	2 seabirds	Eggs	40	13.2-17.1	38
	Zooplankton spp.	Whole	103	3.8-19.3	40
	Spiny dogfish	Muscle / Liver	40	3.4-20	42
nation dataset	3 whales	Skin / Blubber	120	4.5-22.2	43
	16 fishes / 10 invertebrates	Muscle / Liver / Gonad / Whole	431	2.5-12.5	35
	30 deepfishes	Muscle	87	4.1-8.5	39
	Mammals / birds	Muscle / Eggs	229	3-20.2	41
	3 whales	Skin / Blubber	120	4.5-22.2	43
crimination ed to dataset	Zooplankton	Whole	—	3.2-10	26
	16 aquatic animals	Muscle	28	3.0-6.9	32
	Spider crab	Muscle / Gonad / Hepatopancreas	46	2.7-51	31
	2 seabirds	Muscle / Liver / Feathers	94	3.2-7.1	34
	2 antarctic fishes	Muscle	20	3.6-7.1	36
	2 cormorants	Muscle / Liver	137	4.4-5.5	37
	Zooplankton spp.	Whole	103	3.8-19.3	40
	3 whales	Skin / Blubber	120	4.5-22.2	43
	Dolphin	Skin	35	3.1-4.5	45
adjusted to dataset	16 fishes / 10 invertebrates	Muscle / Liver / Gonad / Whole	431	2.5-12.5	35
	Zooplankton spp.	Whole	103	3.8-19.3	40
	Mammals / birds	Muscle / Eggs	229	3-20.2	41
	Spiny dogfish	Muscle / Liver	40	3.4-20	42
	3 whales	Skin / Blubber	120	4.5-22.2	43
	8 aquatics birds	Eggs	175	4-17	44
crimination	7 freshwater fishes	Muscle	116	3.2-8.2	27
	16 fishes / 10 invertebrates	Muscle / Liver / Gonad / Whole	431	2.5-12.5	35
	Mammals / birds	Muscle / Eggs	229	3-20.2	41
	Spiny dogfish	Muscle / Liver	40	3.4-20	42
	3 whales	Skin / Blubber	120	4.5-22.2	43
	Shrimp	Hepatopancreas	138	—	28
crimination ation dataset	Seabass	Muscle / Liver	1256	4-19	30
	Zooplankton spp.	Whole	120	4.8-17.8	33
	2 antarctic fishes	Muscle	20	3.63-7.14	36
	2 cormorants	Muscle / Liver	137	4.4-5.5	37
	30 deep fishes	Muscle	87	4.1-8.5	39
	Zooplankton spp.	Whole	103	3.8-19.3	40
	Dolphin	Skin	35	3.1-4.5	45

Tissue	n	Lipid content (%) dw	BULK			LIPID-FREE		
			$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	C:N	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	C:N
W	39	2.8±1.8	-17.3±0.4	12.5±0.8	3.16±0.09	-17.1±0.3	12.7±0.8	3.09±0.04
R	39	11.4±5.2	-18.7±0.5	13.3±0.8	4.10±0.33	-17.8±0.4	13.4±0.8	3.53±0.13
L	39	15.5±7.6	-19.0±0.7	11.4±0.8	5.00±1.00	-17.5±0.4	11.5±0.8	3.92±0.41
G	21	10.4±6.6	-17.8±1.0	11.1±0.6	3.52±0.61	-16.8±0.5	11.2±0.6	3.05±0.31
W	42	2.1±0.5	-17.1±0.3	11.1±0.9	3.11±0.05	-17.0±0.3	11.1±0.8	3.06±0.04
R	41	9.9±2.9	-18.5±0.5	12.3±0.9	3.96±0.19	-17.7±0.4	12.3±0.9	3.51±0.14
L	42	15.1±5.5	-18.9±0.6	10.4±1.0	4.68±0.76	-17.6±0.4	10.5±1.0	3.74±0.37
G	31	11.9±2.5	-18.4±0.6	10.4±1.1	3.65±0.43	-17.1±0.6	10.5±1.1	3.03±0.31
W	48	2.3±1.1	-16.9±0.4	11.7±1.0	3.15±0.16	-16.8±0.4	11.9±0.9	3.06±0.03
R	48	11.7±3.5	-18.4±0.5	12.8±1.0	4.11±0.33	-17.5±0.4	12.9±1.0	3.53±0.13
L	48	19.6±11.2	-19.1±1.1	10.9±0.9	5.10±1.31	-17.3±0.5	11.0±1.0	3.68±0.28
G	30	12.5±7.6	-18.0±1.1	10.7±0.7	3.67±0.95	-16.6±0.6	10.8±0.7	2.98±0.23

1st lipid-extraction	2nd lipid-extraction
18.6	2.1
-17.4±0.5 ^a	-17.4±0.4 ^{ab}
11.3±0.6	11.3±0.7 ^a
4.25±0.30 ^a	5.74±0.32 ^{ab}

$\Delta\delta^{15}\text{N}$			$\Delta\delta^{13}\text{C}$		
Squares	F value	Pr(>F)	Mean Squares	F value	Pr(>F)
203	2.69	NS	12.036	3170.06	p<0.001
2988	3.79	p<0.05	0.184	48.39	p<0.001
3234	2.41	NS	0.008	2.09	NS
244	0.71	NS	0.027	7.15	p<0.01
3423			0.004		

Table 5.

Tissue	McConnaughey & McRoy ^[25] [eq1]		Post et al. ^[32] [eq2]		Logan et al. ^[35] [eq3]
	<i>D</i>	<i>I</i>	<i>a</i>	<i>b</i>	<i>D</i>
W	6.4±0.9	-0.012±0.004	1.97±0.13	-6.05±0.42	6.6±0.38
R	6.2±0.4	0.001±0.009	1.01±0.07	-3.22±0.26	6.6±0.09
L	8.1±0.3	-0.018±0.007	0.76±0.02	-2.18±0.12	6.9±0.1
G	7.4±0.4	0.028±0.008	0.59±0.08	-0.817±0.29	6.8±0.16

Table 6.

		Adjusted parameters				Literature parameters			
		W	R	L	G	W	R	L	G
McConnaughey & McRoy ^[25] [eq1]	Good predictions (%)	88	89	86	89	0	0	0	0
	Mean $\Delta\delta^{13}\text{C}_{\text{error}}$ (‰)	-0.001±0.035	-0.001±0.046	-0.001±0.068	0.004±0.055	0.3±0.03	-1.3±0.03	-1.6±0.05	-1.7±0.04
	Max $\Delta\delta^{13}\text{C}_{\text{error}}$ (‰)	0.6	0.9	1.6	1	1.9	2	2.8	2.2
Post et al. ^[32] [eq2]	Good predictions (%)	88	91	90	89	0	0	92	0
	Mean $\Delta\delta^{13}\text{C}_{\text{error}}$ (‰)	-0.002±0.029	0.0±0.045	-0.005±0.076	0.004±0.091	-0.4±0.02	-0.2±0.03	0.0±0.06	-1.1±0.07
	Max $\Delta\delta^{13}\text{C}_{\text{error}}$ (‰)	0.5	1.2	1.3	1	0.8	1	1.5	1.9
Fry ^[27] [eq3]	Good predictions (%)	87	54	16.9	10.2	0	0	0	0
	Mean $\Delta\delta^{13}\text{C}_{\text{error}}$ (‰)	-0.002±0.028	-0.047±0.029	-0.184±0.04	-0.278±0.036	-0.1±0.03	-0.9±0.05	-1.9±0.16	-1.4±0.09
	Max $\Delta\delta^{13}\text{C}_{\text{error}}$ (‰)	0.5	1.2	1.4	1.6	0.67	1.8	5.2	3.2

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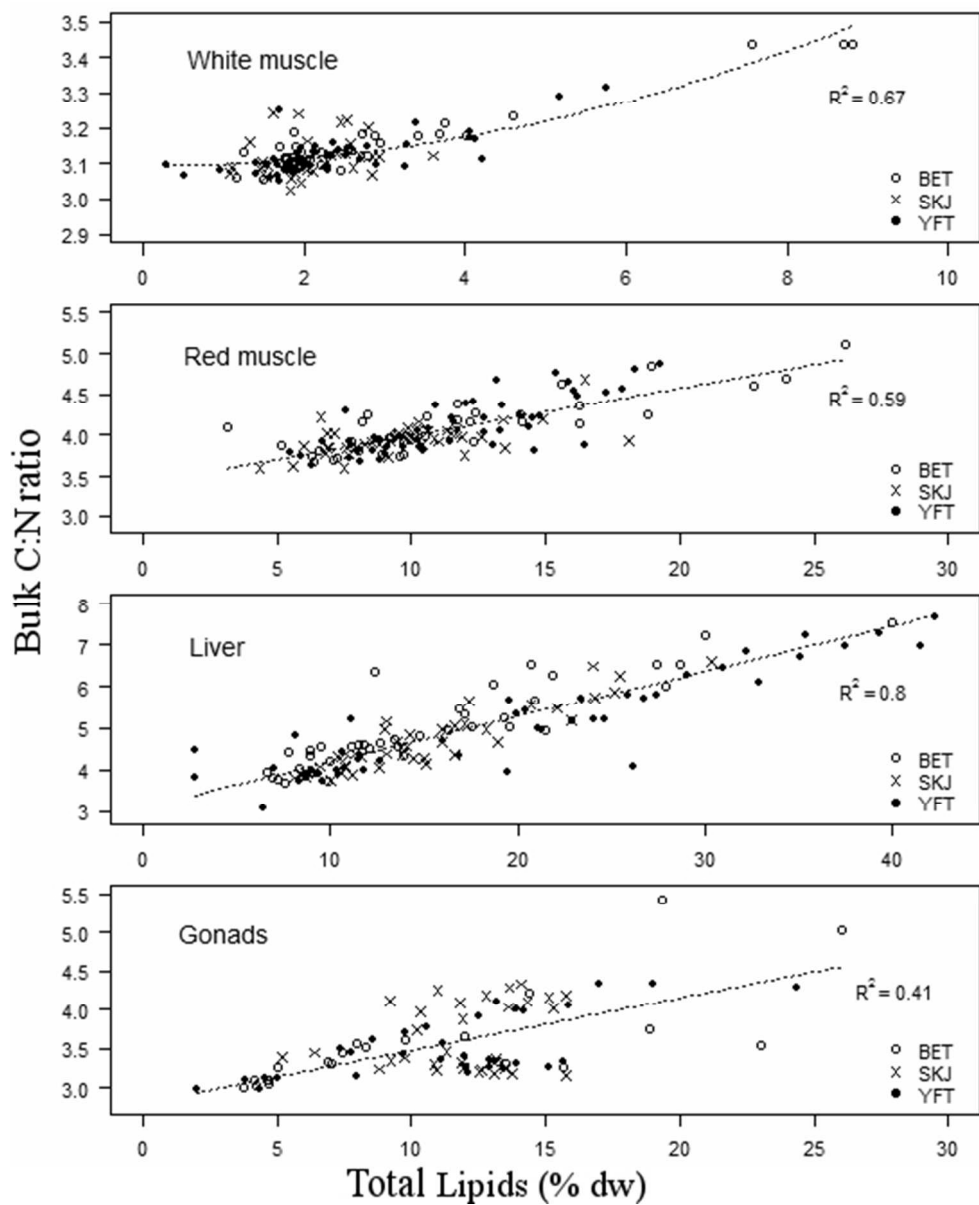


Figure 1. Relationship between bulk C:N ratio and total lipid content (% dw) for each tissue. A curvilinear model was significant in white muscle only. Coefficients of determination (R^2) are given.

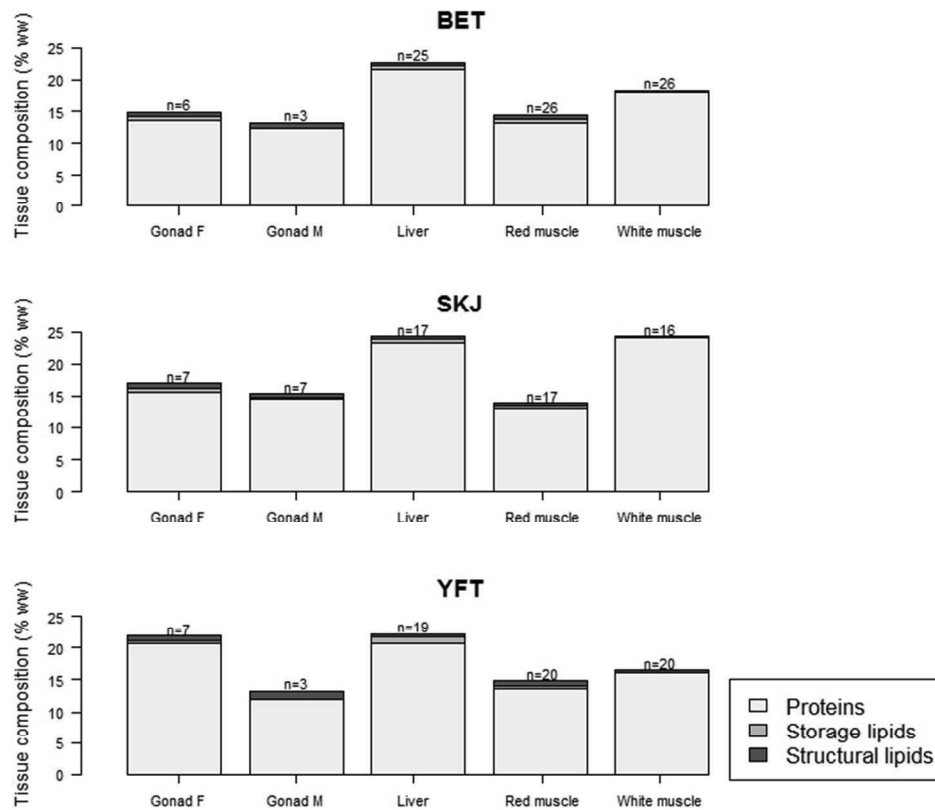


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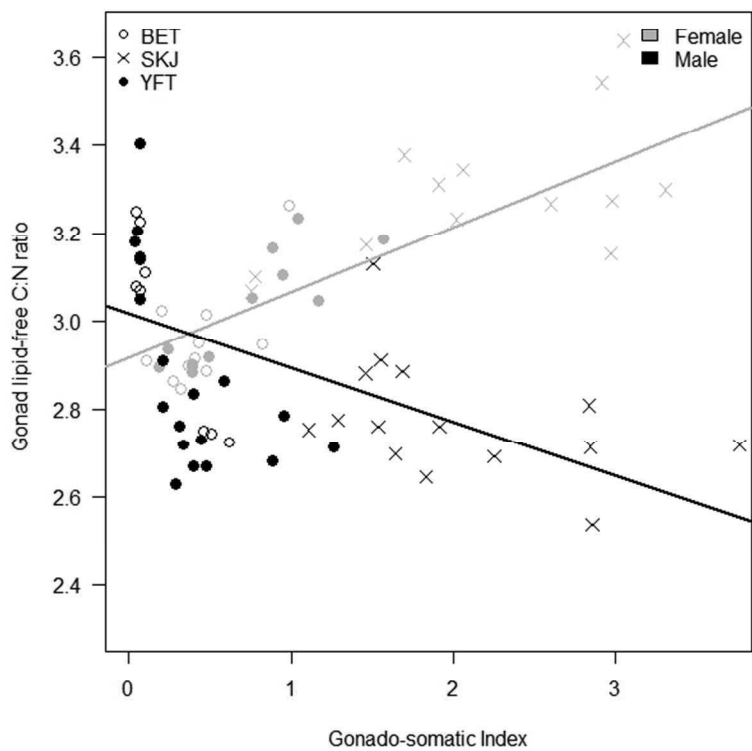


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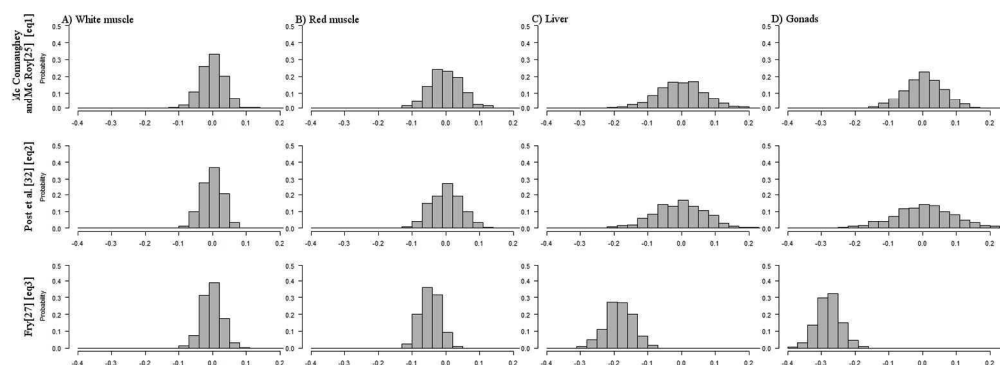


Figure 4. Distributions of the $\Delta\delta^{13}\text{C}_{\text{error}}$, i.e. the differences between the lipid-corrected and observed lipid-free $\delta^{13}\text{C}$ values. Lipid-corrected values were predicted from the three lipid-normalization models using a cross validation procedure that was repeated 500 times. $\Delta\delta^{13}\text{C}_{\text{error}} = \delta^{13}\text{C}_{\text{corrected}} - \delta^{13}\text{C}_{\text{lipid-free}}$
592x210mm (72 x 72 DPI)